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The fine-tuning of thermosensitive and degradable polymer micelles for enhancing intracellular uptake and drug release in tumors

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ABSTRACT

Focusing on high temperature and low pH of tumor tissue, we prepared temperature and pH responsive poly(N-isopropylacrylamide-*co*-N,N-dimethylacrylamide-*b*-lacitde) (PID_{118}-*b*-PLA_{59}) and poly(N-isopropylacrylamide-*co*-N,N-dimethylacrylamide-*b*-ϵ-caprolactone) (PID_{118}-*b*-PCL_{60}) diblock copolymers with symmetric hydrophobic blocks by the reversible addition-fragmentation chain transfer (RAFT). The corresponding dual functional polymeric micelles were fabricated by dialysis methods. Their well-defined core-shell structure was characterized by ^{1}H NMR in D_2O and further confirmed by TEM. Their structural and physical chemistry properties such as diameters (D), core corona dimension (R_{core}, R_{shell}), distribution (PDI), M_w, aggregation number (N_{agg}), second virial coefficient (A_2), critical micellization concentration (CMC) and z-potential were firstly systemically investigated by dynamic and static laser light scattering. The volume phase transition temperature (VPTT) was around 40 °C above which the intracellular uptake of adriamycin (ADR) was significantly enhanced. Both flow cytometry and fluorescent microscopy showed that the ADR transported by these micelles was about 4 times higher than that by the commercial ADR formulation Taxotere®. In vitro cytotoxicity assay against N-87 cancer cell and confocal laser scanning microscopy (CLSM) also confirmed such promoting efficiency. In addition, it was interesting to find that cell surviving bounced back as $T = 42$ °C due to the inter-micellar aggregation. The well clarified mechanism strongly support that our finely tailored dual functional core-shell micelles are potent in enhancing cellular uptake and drug release.

1. Introduction

The specific nano scaled multi-assemblies, polymeric micelles, comprising amphiphilic block copolymers were intensively investigated due to their structural stabilization in aqueous media. The hydrophobic core has high capacity in hydrophobic anticancer loading via the similar-to-similar interaction. While its densely packed corona forming hydrophilic polymer chain can protect nano micelle system from the reticuloendothelial system (RES) by reducing the interaction with serum proteins [1–6]. It is known that the ideal nano-based drug delivery system (DDS) should be able (a) to specifically accumulate in the required organ or tissue and then (b) penetrate inside target cells, delivering its load (drug or DNA) intracellularly [7–9]. For the accumulation of carrier system in the required organ or tumor tissue, some targeting moieties such as antibody, folic acid, transferrin and peptide were linked to the particle surface, which can specifically bind with cancer cell [10–12]. In order to overcome the intracellular releasing, many novel simulative-responsive micellar systems were intensively investigated recently including the temperature, light, pH and ultrasound signals [13–16]. We and other groups independently developed thermoresponsive polymer micelles comprising diblock copolymers of poly(N-isopropylacrylamide) (with a lower critical solution temperature, LCST ~ 32 °C) and various hydrophobic segments such as poly(butyl methacrylate) and poly(D,L-lactide) as systems to improve the cancer chemotherapy. These drug-loaded thermoresponsive micelles containing hydrophilic PNIPAM corona successfully accumulated in tumor tissue and controlled drug release which was proved by the in vitro cytotoxicity against endothelial or various cancer cells with applied temperature changes [17–19].

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Up to now, the relation between polymer composition, micellar intelligent properties and in vitro cytotoxicity was not so clear yet. This maybe lack understanding of the detail relation between *in vivo* tumor signal (needs) and in vitro micellar physical chemistry properties (seeds). Thus, much attention should be focused on the tumor's characteristics when we design and fabricate intelligent nano carriers. While the tumor cell will endlessly grow and fast metabolism once mutating, which makes the tumor tissue very different from the normal tissue with some special properties such as high temperature (~40 °C), low pH (5.3) and anomalistic vessel (intercellular gap junction = 300–700 nm) [20–22]. Okano's group has successfully developed a novel thermosensitive core-shell micelles and found that the interaction between cell and micelle can be tuned by changing temperature. As $T >$ LCST, the cell uptake was enhanced [23–26]. On the other hand, the drug delivering profile strongly depends on micellar physico-chemistry properties including size, structure and its intelligent response, which is dominated by the block copolymer's composition. Fortunately, the reversible addition-fragmentation chain transfer (RAFT), a powerful tool for the tuning the block copolymer composition, can be used to tailoring the micellar structures as wanted [27–35].

On this sense, firstly, we use the conventional RAFT to prepare poly(N-isopropylacrylamide-*co*-N,N-dimethylacrylamide) thermosensitive blocks. Then the hydrophobic biodegradable blocks PLA and PCL are linked to the thermosensitive blocks by ring-open polymerization. By this way, the volume phase transition temperature (VPTT) was tuned targeting to the temperature of tumor tissue (~39 °C). The degradable PLA/PCL responds to the endosomal acid environment (pH ~ 5.3). The relation between such dual-functional intelligent properties micelles and the intracellular uptake, cytotoxicity profile and trigged drug release was also clarified in this study.

2. Materials and methods

2.1. Materials

N-Isopropylacrylamide (NIPAM) was kindly provided by Kojin (Tokyo, Japan) and purified by recrystallization from *n*-hexane. N,N′-dimethylacrylamide (DMAAm) (Wako Pure Chemicals, Osaka) was distilled under reduced pressure. D,L-Lactide (LA) (Tokyo Chemical Industry, Tokyo) was recrystallized from ethyl acetate. ϵ-Caprolactone (CL), N,N-Dimethylformamide (DMF), 2,2′-azobis[2-methyl-N-(hydroxyethyl)]propionamide (VA-086), dimethyl sulfoxide (DMSO), tetrahydrofuran (THF), N,N-dimethylacetamide (DMAc), xylene, diethyl ether, sodium thiosulfate ($Na_2S_2O_4$), Na_2HPO_4, NaH_2PO_4, hydrodated phosphotungstate (PTA), 4% paraformaldehyde, tetramethylrhodamine-5-carbonyl azide (Rhodamine, or TAMRA) and highly purified 1,4-dioxane were obtained from Wako Pure Chemicals and were used without further purification. Maleimide (Mal) (Aldrich, St. Louis, MO), 2-hydroxyethylamine (Kanto Chemical, Tokyo), tin(II)2-eth-ylhexanoate (Aldrich), 2-ethanolamine (Kanto Chemical Co. Inc., Tokyo, Japan), Dulbecco's phosphate-buffered saline (PBS) and Albumin from bovine serum (BAS, minimum 96%, Sigma) and the commercial ADR formulation Taxotere® (Changhai Hospital, Shanghai) were used as received. A reversible addition-fragmentation chain transfer agent, 2-[N-(2-hydroxyethyl) carbamoyl] prop-2-yl dithiobenzoate (HECPD), was prepared according to previous publications [17,24,25]. Water used in this study was purified by a Milli-Q Synthesis A10 system (Millipore, Billerica, MA) unless otherwise mentioned.

2.2. Polymer synthesis

2.2.1. PNIPAM-*c*-DMAAm random copolymers synthesis

The PNIPAM-*c*-DMAAm random copolymer with 118 co-monomer unit (PID_{118}-*OH*) was prepared by the conventional RAFT polymerization. Shortly, NIPAM 13.6 g (3 M), DMAAm 3.96 g (1 M), HECPD 0.17 g (15 mM) and VA-086 34 mg (3 mM) were mixed and dissolved in 40 mL 1,4-dioxane. The solutions were degassed under reduced pressure by 3 times freeze-pump-thaw cycles. The polymerization was carried out in pre-heated 70 °C water bath for 7 h. After polymerization, polymer was precipitated two times in excess diethyl ether, following by thorough drying under vacuum. The obtained pink copolymer with –OH and dithiobenzoate terminal group PID_{118} was stocked at low temperature as the macro-CTA. The synthesis process was shown in Scheme 1 (①).

2.2.2. Aminolysis and conversion of polymer termini

Scheme 1 (②) shows the conversion of dithiobenzoate end group to the hydrophilic amine group. A propositional amount of PID_{118}-*OH* (0.015 mmol), 2 mol equivalents $Na_2S_2O_4$ and 40 mol equivalents of maleimide (*vs* terminal groups) were dissolved in 5 mL THF pre-deoxidized by N_2 for 1 h. 2-Ethanolamine (20 mol equivalents *vs* terminal dithiobenzoate groups) in 1 mL pre-deoxidized THF was slowly dropped into the polymer solution under N_2 bubbling following by 20 h reaction at room temperature. After reaction, the solution was dialyzed against Milli-Q water (resistivity of 18.3 MΩ, Millipore, CA) using the dialysis membrane (MWCO 1000, Spectra/Pro 6, Spectrum Medical Industries, Los Angeles, CA) until complete removal of the unreacted chemicals and the organic solvent THF. Then the final white product polymers with maleimide end group were recovered by freeze-drying [17,25].

2.2.3. PID_{118}-PLA_{59} and PID_{118}-PCL_{60} diblock copolymers synthesis

The block copolymers with similar hydrophobic block length (PID_{118}-*b*-PLA_{59} and PID_{118}-*b*-PCL_{60}) were synthesized using PID_{118} as the macro-CTA as shown in Scheme 1 (③). In the conventional ring open polymerization, proportional calculated amount of monomers were weighted, the PID_{118} (0.75 g, 6×10^{-5} mol), D,L-lactide (0.5 g, 10 mM) or ϵ-caprolactone (0.48 g) were mixed in a 25 mL nash-flask connected with a condenser. The mixed powder was dried at 50 °C under vacuum for about 2 h. Then 4 mL pre-degassed xylene was injected into each flask under Ar. The imitator tin(II)2-eth-ylhexanoate (40 mg) was pre-dissolved in about 2 mL pre-degassed xylene was injected in to the monomer solution under Ar. The polymerization was carried out at 150 °C for about one day. After polymerization, the solutions were distillated at 50 °C under vacuum to remove the xylene and re-dissolved in 5 mL THF. The precipitation was removed by centrifugation. The up clear solution was collected and precipitated in excess diethyl ether two times. The pale powder was obtained under vacuum drying.

2.2.4. Conversion of PID-PLA/PCL termini group to fluorescence group

The fluorescence marker tetramethylrhodamine-5-carbonyl azide (Rhodamine, or TAMRA; M_w, 567 g/mol) was reacted with the –OH terminal group on the hydrophobic PLA or PCL block. The reaction was: block copolymer and TAMRA (5 mol equivalents to OH termini) was dissolved in degassed DMF (3 mL), then reaction was carried out at 80 °C for 5 h at dark conditions. After the reaction, polymer solutions were dialysized against the methanol using the dialysis membrane (Spectra/Pro 6, MWCO 1000) (Spectrum Laboratories, Rancho Dominguez, CA) for about 4 days. The solution in membrane was continued to dialysis against di-water for about 3 days to remove the un-reactive impurities completely. The fluorescent termini polymers were recovered by freeze-dry. The reaction was shown in Scheme 1 (④).

2.2.5. Characterization of the block copolymers

The number averaged molecular weight (M_n) was converted from the UV (UV–vis spectrometer, V-530, Japan Spectroscopic Co., Ltd, Tokyo, Japan) absorption of dithiobenzoate group at 500 nm. The polydispersity (M_w/M_n) was obtained form GPC (Tosoh, SC-8020). The compositions were characterized by ^{1}H NMR (400 MHz, Varian Inc.) using chloroform-D ($CDCl_3$) as solvent. The properties of these block copolymers were summarized in Table 1.

2.3. Fabrication and characterization of micelles

2.3.1. Fabrication of micelles

The micelles were prepared by dialysis method as mentioned in our previous publications [17,24]. About 10 mg diblock copolymer was dissolved in 1.5 mL DMAc, then dialyzed against Milli-Q water using the dialysis membrane (MWCO 1000, Spectra/Pro 6, Spectrum Medical Industries, Los Angeles, CA) at 4 °C for about 3 days with regular water changing. Then the solutions with calculated concentration 1.8 mg/mL were stocked for further characterization.

2.3.2. Chemical structure analysis by ^{1}H NMR

About 10 mg PID_{118}-*b*-PLA_{59} and PID_{118}-*b*-PCL_{60} was dissolved in DMAc, then dialyzed against Di-H_2O for about 2 days with regular water changing. The collected micelle solution was freeze-dried for one day to obtain micelle powder. Then add about 1.5 mL D_2O into the micelle powder with gentle shaking. The micelle structure was characterized by ^{1}H NMR (400 MHz, Varian Inc.). The ^{1}H spectrum of each sample was recorded for analyzing the signal of PLA in $CDCl_3$ and D_2O.

2.3.3. Size distribution and molecular weight by LLS

The hydrodynamic diameter (D) and size distribution (PD.I.) were determined by ZetaSizer (Nano-ZS, Malvern Instruments, Worcestershire, UK) equipped with a He-Ne laser (633 nm) at a scattering angle 173° (micelle concentration: 1.8 mg/mL). The molecular weight (M_w) was obtained from the static laser light scattering (SLLS) by the Debye plots. Before the SLLS, the increscent of the refractive index dn/dc_p was determined by the double bean differential refraction meter (DMR-1021, Ostuka Electronics, Tokyo Japan) [36]. Four concentrations (ca. 0.225, 0.45, 0.9 and 1.8 mg/mL) were prepared for the SLLS experiment.